THE EFFECTS OF THE EXPRESSION LEVELS OF CELLOBIOSE TRANSPORTER AND β-GLUCOSIDASE ON ETHANOL FERMENTATION

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ABSTRACT

Recently, cofermentation of cellobiose and xylose in yeasts has been reported. It is considered as one of the most innovative strategies to enhance bioethanol production from lignocellulosic fermentation. Through cofermentation with cellobiose and xylose, it is achieved to utilize most abundant two sugar substrates in lignocellulosic materials at the same time and to enhance xylose utilization by yeast through boosting cellobiose catabolism in yeast. However, cellobiose utilization by yeast to produce bioethanol has not been fully understood yet. Due to cellobiose fermentation in yeast requires the introduction of two essential enzymes such as cellobiose transporter and β-glucosidase, the ratios between those two enzymes can be a significant factor on cellobiose fermentation.

In order to assess the effects of expression level of cellobiose transporter (CDT) and β-glucosidase (β-GL), in this study the copy number variation of plasmids is used to assess contributions by CDT and β-GL on cellobiose fermentation. Four different transformants contain different combinations of copy numbers in CDT and β-GL; MTMβ, MTSβ, STMβ and STSβ. The patterns of cellobiose fermentation by the transformants were evaluated. The engineered strain, MTMβ showed the best fermentation phenotypes relevant to cellobiose fermentation. The productivity of the engineered strain showed 0.588 g/h·L, and final yield of ethanol is 0.413 g/g.

Additionally, this study suggests that higher expressed CDT has more critical influence on cellobiose fermentation. When I compared MTSβ and MTMβ, I observed detrimental contribution of insufficient β-GL on cell growth and ethanol fermentation. MTSβ strain showed 1.8 fold of cellodextrin accumulation and takes more 46 hours to finish cellobiose fermentation, compared to STSβ. However, when I compared STMβ and STSβ, I observed beneficial contribution on cell growth and ethanol fermentation. MTMβ showed

0.78 fold of cellodextrin accumulation and takes less12 hours to finish cellobiose fermentation, compared to STMβ.

Taken these two observations together, it is suggested that in the case of ensuring sufficient quantities of β-GL, overexpression of CDT provide beneficial effects on cellobiose fermentation.

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Chapter 1

Introduction

1. 1 Current State

Recently, the high price of oil combined with a growing interest in the development of alternative transportation and liquid fuels have spurred a rapid expansion of biofuel research (EPAct 2005). Also, the environmental consequences from the use of fossil fuels is widely documented and reported which has led to an active search for renewable and cleaner biofuels (M O'hare, 2009). A biofuel should provide substantial benefits to both the consumer and producer to become a practical and cost effective alternative to fossil fuels. To compete economically with fossil fuels, biofuels must be developed so that sufficient quantities can influence the energy demand while having a net energy gain compared to the input energy required to produce them (Hill, 2006).

At present, corn bioethanol has been accepted as a dominant biofuel in the U.S. for the past few decades and is intensively supported by the U.S. federal government and many states in recent days (Schnepf, 2006). However, as government and scientific researches pointed out disadvantages of corn grain ethanol in many areas (Searchinger, 2008), U.S. government tried to seek better options for biofuels. One of the emerging biofuels is lignocellulosic bioethanol. Lignocellulosic ethanol has various and unique advantages over corn grain ethanol in terms of capacity to produce, beneficial influence on both environment and economy, and being free from many controversial issues.

For a practical substitute to corn grain ethanol, lignocellulosic ethanol must resolve

some major obstacles to utilizing lignocellulosic biomass in an efficient way. Specifically, some of the prominent obstacles include deconstruction feedstock, sugar fermentation and crosscutting researches for bioengineering. Since ethanol industries still heavily depend on corn grain and lignocellulosic biomass based ethanol industries is only at initial stage, overcoming these obstacles is essential part of lignocellulosic ethanol production to make a considerable dent in gasoline consumption in the near future.

Recently, the progress of scientific researches for lignocellulosic ethanol has been impressive. Especially, the development of the biological and metabolic engineering fields has been growing very rapidly. One of the notable research topics from lignocellulosic ethanol is cofermentation. The concept of cofermentation is simply utilizing different sugar sources at the same time. However, because the two major substrates which are the most abundant in lignocellulosic biomass are composed of cellulose portion and xylan portion, cofermentation is considered as an innovative strategy to improve efficiency in sugar fermentation dramatically.

1.2. The Purpose of This Research

Even though the introduction of utilizing cellobiose (a dimer form of sugar molecule from cellulose) as a sugar source opens many possibilities to enhance sugar fermentation efficiency, fermenting cellobiose as a sugar source still remains uncovered inside of cellobiose utilization. Also, there are some possibilities to enhance productivity of cellobiose fermentation for better cofermentation. Because cellobiose fermentation depends on the efficiency of the given CDT and β-GL, fermentation productivity is considerably affected by the combination of those two factors'

expression level. In order to understand more in depth about efficient cellobiose fermentation, it is needed to evaluate the effects of the expression level of the two main enzymes. This will reveal the best combination of those genes for maximizing productivity and the most optimal combination of those genes.

The specific objectives are as follows:

- To investigate the fermentation patterns of four different engineered strains
- To compare the four fermentation profiles of those engineered strains
- To evaluate important factor or combination on cellobiose fermentation

Chapter 2

Literature Review

2.1 The Current State of Fossil Fuel Utilization and Biofuels in U.S.

The United States' desperate needs for a liquid fuel replacement for fossil oil in the near future provoked 2006 State of the Union address at which the president Bush announced the new Advanced Energy Initiative (AEI) to overcome U.S.'s dependence on foreign energy source. Its goal was to reduce the national dependence on imported petroleum fuel by accelerating the development of domestic and renewable alternative fuels. After that, two major departments of energy (DOE) offices are targeting to advance biofuel researches: The office of biological and environmental research (OBER) within the office of science and the office of the biomass program (OBP) within the office of energy efficiency and renewable energy. These offices have been aiming to support a substantial and sustainable expansion of biofuels so far now, and the concrete target of the displacement of fossil fuels is estimated up to 30% of the nation's current fossil fuels use by 2030 (EPActs 07).

The U.S. Energy Policy Act of 2005 has established forceful short term targets for ethanol production. An important provision requires 7.5 billion gallons per year of renewable fuel by 2012, and for 2013 and beyond the required volume should include 250 million gallons of cellulosic ethanol; the production goal is called a renewable fuel standard (RFS). Additionally, to legislative mandates, the Biomass R&D Act of 2000 acted to establish the Biomass Research and Development Technical Advisory Committee. It set a goal requiring biofuels to meet 20% of transportation fuel by 2030. Moreover, the National Commission on Energy Policy requires producing the

equivalent of nearly 7.9 million barrels of oil per day by 2050, or 50 % of total fossil oil us in the transportation, or 3 times as much as import from the Persian Gulf alone. Furthermore, the Energy Independence and Security Act of 2007 mandates 36 billion gallons of renewable fuels by 2022, with the caveat that 21 billion gallons must be produced from non-corn feedstock.

These kinds of biofuel legislatives seem like leading tangible improvements in that the U.S. imports of crude oil and total petroleum products from other countries gradually have diminished from 2005, and also in 2010 the U.S. imported fossil fuels and total petroleum products is now decreased to 42 billion gallons, which is 10 billion gallon slower than 2005 (EIA 2010). However, considering these consequences are not even close to the original expectations, and biofuels constitute the only renewable liquid fuels that can be integrated readily with petroleum based fuels and infrastructure transports, it is obvious that biofuels as a renewable alternative to fossil fuels would be and should be increasingly becoming a focus on a development.

Biofuels can be a strong strategy for U.S. current state to ensure the national security. Because, even though U.S. accounts for almost 25% of global oil consumption, it holds only 3% of global oil reservoir (Leiby, 2007), its much reliance on imported fossil fuels can lead to a critical threat to the country's integrity. However, unlike fossil fuels, biofuels such as utilizing biomass as a source of energy are an attractive option to ensure national security in that biomass is domestic, secure and abundant feedstock.

Lastly, the encouragement of biofuels is expected to help stimulate beneficial effects on industrial fields, such as creating more jobs, ensuring more profits to both farmers and the government, and fostering the growth of domestic economy (Parcell and Patrick, 2006; Warner, 2007; Cavaney, 2007). For instance, in 2004 the ethanol

industry created 147,000 jobs in all the economy sectors and earned more than $2 billion of tax revenues to federal and local governments (RFA2005).

However, in order to be a practical alternative for fossil fuels, a biofuel should provide not only national security, economical profits, but also enough quantity of production to meet the expected demand of biofuel. In 2005, it was reported that more than 1 billion dry tons of biomass is annually required to displace at least 30% of the nation's current consumption of liquid transportation fuels (Perlack, 2005; Breaking the chemical and engineering barriers to lignocellulosic biofuels, Roadmap 2007). And also it was reported that approximately 2 billion acres of land area in U.S. could be served as biofuel production and 1.366 billion dry tons of biomass could be derived from those areas (Perlack, 2005).Thus, it is required that enough land to provide the needed large-scale supply of biomass and it is believed that biofuels enough sustain to produce at the scale needed to make a real difference in transportation consumption of fossil fuels.

Generally, biofuels is a type of fuels derived from any form of biomass and it can take any form of many different fuels. In U.S. there are two major widely received biofuels to meet those conditions for alternative fuels, which are mentioned above. One is bioethanol and the other is biodiesel (Pimentel and Patzek, 2005). Those have been considered as alternative fuels for a few decades, which are able to displace gasoline and diesel, because of their practical and economic advantages over other biofuels. However, the portion of biodiesel is not as big as that of bioethanol yet and bioethanol has preoccupied most of biofuel markets. In addition, corn grain bioethanol comprises a large portion of bioethanol now. In practice, the facilities and farms in major corn-producing areas have increased as part of those efforts biofuel production. Especially in 2007, corn plantings in the U.S totaled 37.9 million ha, 19% increase over the previous

year which is the record high since 1946 (Pimentel, 2009).

2.2 Corn Grain Biofuels And Limitations

President Obama has not given a specific guideline for the future plan of ethanol yet. On the White House website, a plan is announced to invest $150 billion to reduce the dependency on fossil oil. Most of legislations on biofuels are still based on those of former President. In order to achieve these goals described in EPActs 05 and 07, with solely corn grain ethanol, it could be rarely attainable. For instance, in 2006, about 71 millions of acres of corn were harvested and only 17% of those domestic corn crops are used for ethanol production, which is equal to 1.7 billion of ethanol and only 0.9% of the gasoline for U.S. transportation (Pimentel, 2003). Furthermore, numerous scientific and economic studies have raised questions that ethanol production does not bring out a positive net energy balance, it is hard to be considered as a renewable energy and also it is not able to lead practical advantage on economy and environment (Kendall, 2009; Somma 2010; Pimentel 2009).

2.2.1 Net Energy Balance of Corn Grain Ethanol

To assess energy balance of corn ethanol is not simply accessible, because it is difficult to cover all the possibilities to involve with ethanol production during whole processes. Thus it is not surprising that many researchers provide different evaluation criteria to calculate NEB of corn ethanol. The most recent research to assess net energy balance of corn grain ethanol which is supported by USDA suggests NEB of corn ethanol could be negative. The corn grain ethanol yield is about 2.5 gallons from a bushel of corn (56 pound or 25.5 kg). The corn yield of corn plant is about 8,590 kg

from one hectare of corn farm. Thus, a hectare of corn grain ethanol can be about 842 gallons of ethanol (Pimentel 2007). For the better evaluation of corn grain ethanol, it is required that with those processes, all the sequential processes, such as full irrigation, fertilization, fermentation and distillation were taken into consideration. Thus, the final cost of ethanol production can be $2.24, which number was already included subsidies from many sources. Whereas the current cost of gasoline is about 63 cents/gallon (USBC, 2001). Therefore, net energy balance (NEB) of corn grain ethanol is negative.

2.2.2 Other Issues on Using Feedstock as a Source of Energy

The attempt to use feedstock as a source of energy has detrimental effects on economy in that a shortage of feedstock to domestic animals can cause dramatic increase of the corn grain. Shifting corn utilization to ethanol and away from other uses, would have severe consequences for other agricultural markets, livestock, food prices and land. In reality, more than 70% of the corn grain is utilized as a feedstock to U.S. livestock (USDA 2001). Furthermore, because currently about 3.7 billion people suffer from malnourishment and its consequences of disease, the current food shortages over the world still demand to continue U.S. exports of corn and other grains for food supply (WHO 2002). For example, U.S. corn and other grain exports have been increasing by three times and by $ 3 billion per a year (USCB 2005).

At a glance, it seems like that bioenergy crops can provide farmers with an important new source of revenue and reduce reliance on government funds for agricultural economy, and also higher for traditional crops and new revenue from bioenergy crop could increase net farm income. However, current ethanol production heavily relies on federal and state government subsidies. Also, ethanol production has

been historically concentrated on a very few large companies. For example, the price of corn ethanol is $7.88 per gallon without federal and states subsidies, and the consumer's price of corn ethanol is $2.62 per gallon with the subsidies (McCain 2003, World watch institute). Furthermore, in 2006 the top ten corn ethanol production companies accounted for about 46% of the total output, even those top producers are not equally distributed. Top producer Archer Daniels Midland makes 4 times of capacity of the second company, which is Bio Energy Corporation (Renewable Fuels Association 2007).

Generally, corn farming has severe impacts on agricultural environments. Firstly, corn farming is able to cause under soil erosion (NAS 2003). Secondly, corn production requires more herbicides and insecticides, and more nitrogen fertilizer than any other crops in U.S. (NAS 2003). Thirdly, considerable amount of irrigation is essential for corn production and large amount of water waste is brought out from corn ethanol production. For instance, the production of corn requires 52,000 tons of insecticides, 735,000 tons of herbicides, and 93 million tons of fertilizer which contains nitrogen, phosphorus, and potassium. Also, 1L of ethanol is produced from 1,700L of fresh water, which includes all the water involved with irrigation, fermentation and distillation. Moreover, 12 liters of water wastes comes from 1L of ethanol production, and it is a major contributor to ground water and river water pollution (NAS 2002, Pimentel 2003). Therefore, Corn grain ethanol production actually increases environmental degradation rather than protect the environment so that it is difficult that corn grain ethanol is considered as a renewable and sustainable biofuels for substitutes of fossil fuels.

2.3 Lignocellulosic Bioethanol and Advantages over Corn Grain Ethanol

2.3.1 The Current State of Lignocellulosic Bioethanol

After these following legislatives from 2005, the ethanol production industries had been experiencing a major boom. The demand of ethanol reaches to 5.4 billions of gallons in 2006, and production will increase even more under the EPAct 07, which mandates to increase the current RFS to 35 billion gallons of renewable biofuels by 2017. However, in order to achieve this goal, ethanol needs to be relied on much more heavily. In reality, with only corn ethanol it would be onerous. Because the goal requires much more corn than U.S. currently grows, both converting more cropland to corn production and switching all corn utilization to only ethanol, not from other uses, would cause severe consequences for livestock and agriculture, and food prices. For examples, to achieve the given goals, it is essential to secure roughly 137 million acres of corn cropland and to shift all the corn products from the cropland to ethanol so as to meet 56.4 billion of ethanol, which is equivalent to only 6% of the liquid fuel of U.S. But since 1950, U.S corn-harvest acreage has never reached 76 million of acre yet (Yacobucci, 2007; Capehart, 2008).Therefore, the amount of gasoline displaced is severely hindered by the availability land for corn crops.

Ethanol import from other countries might be an easy approach for enough supply of ethanol. For instance, as the world's largest sugar producer, Brazil has been exporting 94 million gallons of ethanol in 2003, to 211 million gallons of ethanol in 2008 (USDC 2009). Secondly, as the future world's largest ethanol producer, China has been providing ethanol at very competitive prices, only $1.65 per gallon (Hong Yanga, 2009), by using cassava for ethanol production. Chinese ethanol production based on cassava has 2 major advantages over corn grain ethanol and sugarcane ethanol; cassava

can grow marginal lands in which corn, sugarcane and wheat cannot grow well (Stambuk, 2008; Yeboah, 2010) and is inedible source. Thus, it is not surprising that China is expected to be the major partner of U.S. ethanol imports in the near future so as to reduce oil dependency. These current circumstances of ethanol production indicate that U.S. have started or will start to increase ethanol import from other countries. Actually, the annual import is 211 million gallon of ethanol in 2008 (USDC 2009).

However, even though ethanol import has some contributions to mitigate dependence on fossil oil, because it does not guarantee the national security, it can be a temporary measure but cannot be the fundamental solution for alternative fuels.

2.3.2 Transition to Lignocellulosic Ethanol

Most of corn grain ethanol in U.S. is consumed in the blended form of gasoline. 3.6% of transportation gasoline in 2006 was substituted by E-10 and E-85 (a blended form of gasoline with 10% and 85% of ethanol content, respectively) (USEIA 2009). However, ethanol for alternative transportation gasoline is seriously impeded by its own intrinsic characteristics. Ethanol has a lower energy per gallon so that transporting vehicles based on ethanol need to be more often refueled than those based on gasoline. Additionally, ethanol is more caustic to a storage tank or pipeline than gasoline so that the cost for shipping ethanol is considerably high, compared with gasoline. Due to these reasons, despite of a large and rapid growth of ethanol industries, ethanol production is intensively concentrated in a small and restricted region where states are able to provide transportation fuels at lower price than other states. About three quarters of ethanol production depends on only five states in U.S.: Iowa, Nebraska, Minnesota, South Dakota and Illinois (Yacobucci, 2007). Therefore, it is necessary for the expanded use of

ethanol all over U.S. to overcome unequal geographic distribution.

First of all, cellulosic ethanol is considered as a certainly promising second-generation biofuel which will have a very competitive price in the near future. Because it is derived from the most abundant cellulose on earth instead of limited cultivated corn starch, it is estimated the price of cellulosic ethanol will be as low as $0.59 to 0.91 per gallon by 2012(assuming mature developed technology) (Greene, 2004; Farrell, 2006). However, at present cellulosic ethanol production costs are considerably high than corn grain ethanol production cost, mainly due to expensive refining processes.

Secondly, non-edible cellulose has economic and ethical advantages over corn grain ethanol. Because cellulose is found from non-edible food plant material such as wood chips or perennial switchgrass, ethanol production from cellulosic biomass is certainly free from ethical and moral issues (Pimentel and Patzek, 2005). Moreover, it will be also able to allow more regions of U.S produce ethanol conveniently, overcoming high-priced shipment problems of corn grain ethanol (Lin and Tanaka 2006).

Thirdly, cellulosic bioethanol has a competitive price. Since cellulosic ethanol has more energy content, compared to corn grain ethanol, this ethanol production requires less cropland than corn based ethanol production (Righelato and Dominick V. Spracklen, 2007).For example, a biomass energy crop from perennial plants such as switchgrass produces up to 500% more renewable energy than energy consumed in its production. It indicates that cellulosic ethanol has positive net energy balance and renewable benefits. Furthermore, cellulosic biomass converts 45% of the biomass energy into biofuels, which is higher number of ratio than corn grain ethanol has (Farrell, 2006). Considering crude oil production converts almost 85% of the biomass energy

into usable fuels, cellulosic bioethanol energy conversion ratio is quite comparable rather than corn grain ethanol.

Taken these together, because the amount of gasoline replaced through corn grain ethanol is restricted by the limitation from marginal output of corn grain, high-priced transportation cost of corn grain ethanol and sparking ethical and moral controversy, it is absolutely inevitable to shift forward advanced, lignocellulosic feedstock for ethanol.

2.4 Strategies to Improve Ethanol Productivity and Efficiency: Cofermentation and Saccharification of Fermentation

Lignocellulosic bioethanol production through utilizing lignocellulosic biomass from the plants such as wood chips and it has been considered as a good alternative solution of fossil fuels. Not only because since lignocellulosic biomass is very abundant in the earth but also bioethanol among various biofuel from utilizing lignocellulosic biomass definitely has many beneficial aspects in that it is able to be completely combusted and not to produce harmful pollutants (Kaylan, 2007; Kendall, 2009). Moreover, because genome sequencing of many popular microorganisms such as *S. serevisiae* producing ethanol quite well was already documented and well-organized, applying these microorganism into ethanol production is able to encourage powerful and efficient manipulation and design of metabolic pathway through genetic engineering in an easy way. Thus the ethanol fermentation has various advantages over other methods and there is still plenty of room for improvement on ethanol production from lignocellulosic biomass through genetic engineering.

For the efficient and profitable utilization of lignocellulosic biomass for biofuel

production, it is required to enhance the technology in both deconstruction of feedstock and fermentation of sugar at the same time. Especially effective sugar fermentation to ethanol does very depend on metabolizing every constituent sugar in the lignocellulosic biomass, primarily glucose and xylose, because the final product of deconstruction of feedstock constitutes cellulose (glucose polymer) and xylan (xylose polymer). Many approaches to enhance sugar fermentation efficiency and productivity were reported already. Also some of them have started to apply their strategies to utilize lignocellulosic biomass for ethanol production into industrial fields. First strategy is co-fermentation (Nakamura, 2008; Ha, 2011). It is an extended fermentation to utilize more than two of sugar sources at the same time rather than proceeds fermentation with a single sugar source. Second one is simultaneous saccharification of fermentation (Galaska, 2010). It is a comprehensive approach to undergo both the hydrolyzation of lignocellulosic biomass and the fermentation of a sugar at the same time.

However, with glucose and xylose cofermentation, xylose fermentation has a practical limitation. For instance, a wild type of *S. serevisiae* cannot ferment xylose as a carbon source, and furthermore the yeast engineered to ferment xylose through metabolic engineering still showed slow rate of xylose utilization. Due to these reasons, it is a common knowledge that co-fermentation with glucose and xylose is not a much favorable and promising fermentation process. Moreover, during the cofermentation with glucose and xylose, glucose is able to repress xylose metabolism and transportation in various ways. For example, xylose uptake occurs around the end of glucose consumption.

After it was reported that cellobiose is able to be metabolized, it provokes various suggestions that can open the possibilities to help increase efficiency and

productivity of cofermentation because of critical advantages of cellobiose over glucose towards cofermentation, which does not plays a role of repressive effects to other sugars. For example, cellobiose can be utilized by engineered *S.serevisiae* without any repression against xylose. Secondly, cofermentation of cellobiose and xylose is able to show much efficient fermentation profiles. Due to cofermentation that helps to produce more ethanol and in relatively short period of time than the results of the fermentation with sole sugar, it is not surprising that cellobiose and xylose cofermentation has synergic effects to facilitate rapid cell growth, and high ethanol productivity and yield.

Chapter 3

Methods

3.1 Strains and Plasmids

S. serevisiae CenPK was used for engineering of cellobiose metabolism in yeast. *Escherichia coli* DH5 (*F-recA endA1 hsdR17 [rK-mK+] supE44 thi-1 gyrArelA)* (Invitrogen) was used for gene cloning and manipulation. Strains and plamids used in this work is described. The primers used for confirming the transformation of expression cassettes containing *cdt-1* and *gh-1* are listed.

3.2 Medium and Cultures

E. coli was grown in Luria-Bertani medium; 50 μg/ml of ampicillin was added to the medium when required. Yeast strains were routinely cultivated at 30˚C in YP medium (10 g/L yeast extract, 20 g/L Bacto peptone) with 20 g/L glucose. To select transformants using an amino acid auxotrophic marker, yeast synthetic complete (YSC) medium was used, which contained 6.7 g/L yeast nitrogen vase plus 20 g/L glucose, 20 g/L agar, and CSM-Leu-Trp-Ura-His(BIO 101) which supplied appropriate nucleotides and amino acids.

3.3 Fermentation Experiments

Yeast cultures were grown in YP medium containing 20 g/L glucose of SC media and 40 g/L of cellobiose of YP media to prepare inoculums for cellobiose experiment. Cells at mid-exponential phase from SC and YP media containing 20 g/L of glucose and 40 g/L of cellobiose were harvested and inoculated after washing twice by

sterilized water. Flask fermentation experiments were performed using 50 ml of YP medium containing appropriate amounts of sugars in 250 ml flask at 30˚C with initial OD_{600} of ~1 or 10 under oxygen limited conditions. All of the flask fermentation experiments were repeated independently. The variations between independent fermentations were less than 8.8%. Fermentation profiles shown in figures are from on representative fermentation.

3.4 Yeast Transformation

Transformation of expression cassettes for constructing xylose and cellobiose metabolic pathways was performed using the yeast EX-transformation kit (BIO 101). Transformants were selected on YSC medium containing 20 g/L glucose. Amio acids and nucleotides were added as necessary. For the construction of cellobiose consuming recombinant *S. serevisiae*, transformation of *cdt-1* and *gh -1* were selected on YSC medium containing 20 g/L cellobiose. Introduction of expression cassettes into yeast was confirmed by colony PCR with specific primers.

3.5 Plasmid Vector Information

Transporter gene (*cdt-1*) is transferred into pRS 415 and pRS 425 plasmid vector, respectively, and β-glucosidase gene (*gh-1*) is introduced into pRS 416 and pRS 426 plasmid vector, respectively in the same way. Those two vectors, pRS 415 and 416, share a common feature in that they represent single copy number plasmids. However, pRS 415 differs from pRS in that the selection marker of pRS 415 is Leucine protein but the selection marker of pRS 416 is Urasil nucleotide. Likewise, both of pRS 425 and 426 vectors have a common property in that both of them are multi copy number

plasmids. However, the selection marker of pRS 425 is different from those of pRS 426. The selection marker of pRS 425 is Leucine protein but those of pRS 426 is Urasil nucleotide.

3.6 Media Information

Synthetic complete (SC) medium comprises Yeast Nitrogen Base (1.7g/L), ammonium sulfate (5g/L), glucose (20g/L) and a complete supplement mixture of amino acid and synthetic defined medium is taken off indicated given amino acids or nucleotide. Yeast Nitrogen Base supplemented a nitrogen source and the added sugar served as a carbon source. Commonly encountered auxothropies is supplemented by a complete mixture of essential amino acids and vitamins. Thus, cultures on SC media combined with cultures on Yeast Nitrogen base without Amino Acids in combination with drop out mixtures can be used to select for auxothrophies as in Yeast Genetics.

Bacto peptone is an enzymatic digested animal protein for the preparation of bacteriological culture media. The nutritive value of Bacto Peptone is largely dependent on the amino acid content that supplies essential nitrogen. Bacto Peptone contains only a negligible quantity of proteoses and more complex constituents. And yeast extract is the water-soluble and autolyzed yeast. It preserves naturally occurring B-complex vitamins for bacteriological use and cell cultures and growth.

Table 1. Constitutes(g/L) of Synthetic complete media and Yeast P media

a) Synthetic complete media

Synthetic Complete Media Constitutes	
Adenine	0.5 g
Alanine	2.0 g
Arginine	2.0 g
Asparagine	2.0 g
Aspartic acid	2.0 g
Cysteine	2.0 g
Glutamine	2.0 g
Glutamic acid	2.0 g
Glycine	2.0 g
Histidine	2.0 g
Inositol	2.0 g
Isoleucine	2.0 g
Leucine	10.0 g
Lysine	2.0 g
Methionine	2.0 g
p-aminobenzoic acid	2.0 g
Phenylalanine	2.0 g
Proline	2.0 g
Serine	2.0 g
Threonine	2.0 g
Tryptophan	2.0 g
Tyrosine	2.0 g
Uracil	2.0 g
Valine	2.0 g

b) Yeast peptone media

Yeast Peptone Media	
Bacto Peptone	10g
Yeast Extract	20g

3.7 Analytical Methods

Cell growth was monitored by optical density (OD) at 600 nm using UV-visible Spectrophotometer (Biomate 5). Glucose, cellobiose, cellodextrin, glycerol, acetate, and ethanol concentrations were determined by high performance liquid choromatography

(HPLP, Agilent Technologies 1299 Series) equipped with a refractive index detector using a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc.). The column was eluted with 0.005 N of H_2SO_4 at a flow rate of 0.6 ml/min at 50˚C. The analysis of cellodextrin in fermentation samples was performed using high performance anion exchange chromatography (HPAEC) analysis. HPAEC analysis was performed with an analytical column for carbohydrate detection (CarCo.). Filtered samples were eluted with a linear gradient from 100% buffer A (100 mM NaOH in water) to 60% buffer B (500 mM of sodium acetate in buffer A) over 70 min. The flow rate of the mobile phase was maintained at 1.0 ml/min.

Chapter 4

Confirmation of Baseline Cell Growth in the Transformation which have Four Different Combination of Plasmids in Copy Numbers of Cellodextrin CDT and β-glucosidase Genes

For the confirmation whether plasmid does transformation into the target strain or not, Urasil and Leucine are used as selection markers. However, it has not been documented that genetic marker play a role in cell growth and fermentation. It is possible that multi copy number of plasmids have advantages to transformants over single copy number of plasmids through making more essential genetic marker than single copy number of plasmids produce. Thus it is important to check whether more amount of genetic marker contributes to cell growth and ethanol fermentation. Therefore, if genetic marker has some beneficial or detrimental influence on cell growth or fermentation, it is hard to conclude that the inserted CDT and β-GL gene are critical factors that determine cellobiose fermentation profiles.

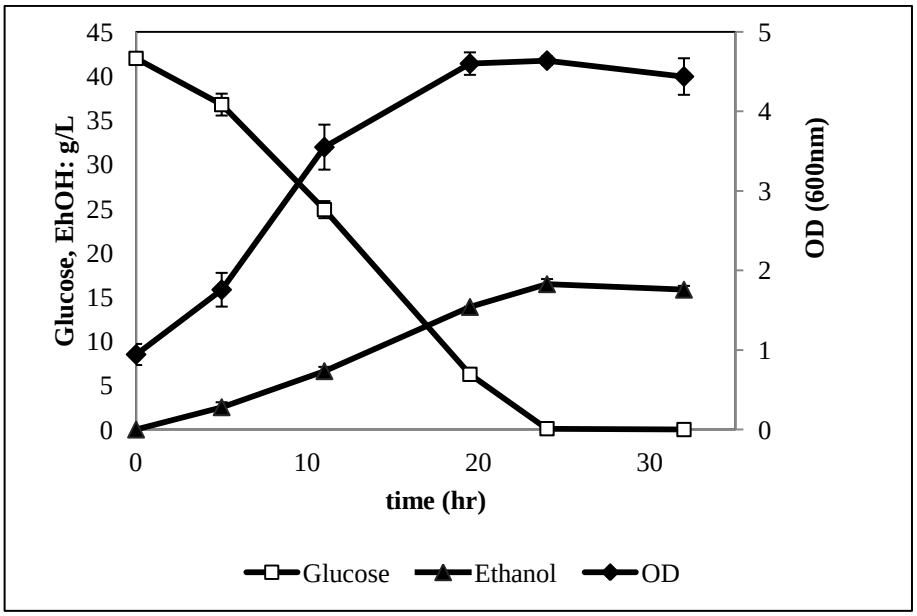
In order to measure how much cell growth and fermentation are affected by plasmids selection markers, the cell growth and ethanol production of those four recombinant transformants were measured under the synthetic defined media which contains 2% of glucose. Synthetic defined media (SC media) has a particular purpose to confirm a complete transformation of plasmids by lacking selection markers such as essential amino acids or nucleotides. SC media cannot provide not fluent amount of nitrogen source but enough amount of nitrogen source for the cell growth. Also glucose serves as a carbon source for the cell growth. In addition, unlike confirmation of

plasmids transformation, Yeast Peptone (YP) median is used as a fermentation medium for the main culture of cellobiose fermentation.

Before the main culture with YP media, I did preliminary experiemnts to make sure that there is no or not significant effects of the selection markers on cell growth or fermentation. For this confirmation, I focused on four features; the capacity to consume glucose within a given time; the cell growth which is measured by OD (600 nm); the volumetric productivity at the maximum state to produce ethanol; a final ethanol yield of the given strain. Because I have hypotheses that the consumption of glucose rate and OD can represent the capabilitiy of the cell growth, and the ethanol productivity and the ethanol yield can indicate the capacity of the strain to produce of ethanol.

4.1 Multi Copy Number of CDT and β-GL

Fig 1. Multi copy number of CDT and multi copy number of β-GL Fermentation Profiles

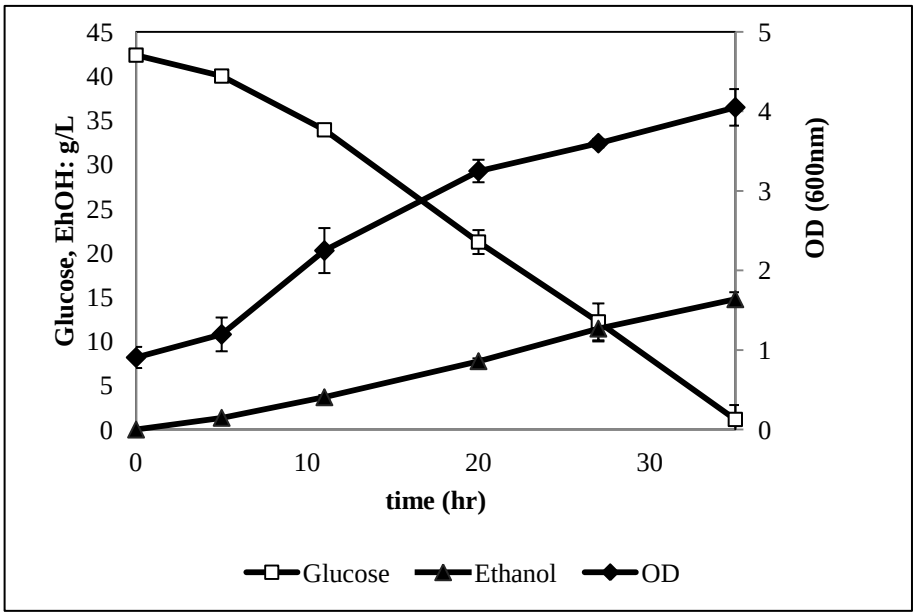


First, MTMβ exhibited a capacity to consume 41.99 g/L of glucose within 24 hours, producing 15.82 g/L of ethanol. Second, the maximum volumetric productivity of glucose fermentation of MTMβ is 0.69 g/h·L. Third, MTMβ is able to reach upto OD 4.60 in 32 hours. Last, the final ethanol yield from glucose of MTMβ ($Y_{Ethanol/Glucose}$) is 0.49.

MTMβ has the fastest cell growth and glucose consumption rates under SCD media and it reached highest OD in a relatively short period of time, compared to all of other three engineered strains. However, despite of higher cell growth rate and glucose consumption rate, it did not exhibit much improved ethanol yield from same amount of carbon source and either did not show much more efficient productivity,

4.2 Multi Copy Number of CDT and Single Copy Number of β-GL

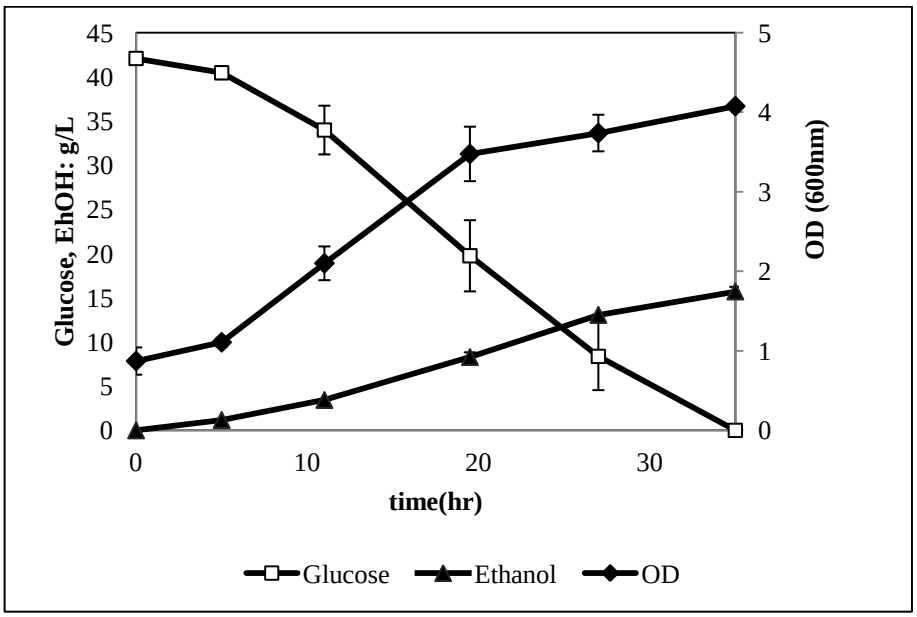
Fig 2. Multi copy number of CDT and single copy number of β-GL Fermentation Profiles



First, MTSβ exhibited a capacity to consume 42.36 g/L of glucose within 35 hours, producing 14.74 g/L of ethanol. Second, the maximum volumetric productivity of glucose fermentation of MTSβ is 0.42 g/h·L. Third, MTSβ is able to reach upto OD 4.48 in 32 hours. Last, the final ethanol yield from glucose of MTSβ ($Y_{Ethanol/Glucose}$) is 0.38**.**

4.3 Single Copy Number of CDT and Multi Copy Number of β-GL

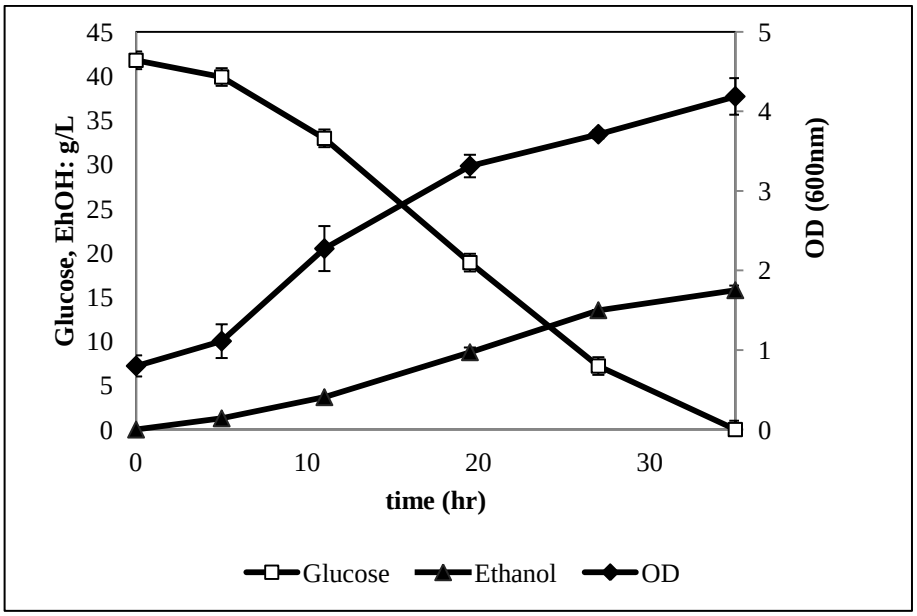
Fig 3. Single copy number of CDT and multi copy number of β-GL Fermentation Profiles



First, STMβ exhibited a capacity to consume 42.07 g/L of glucose within 35 hours, producing 15.71 g/L of ethanol. Second, the maximum volumetric productivity of glucose fermentation of STMβ is 0.48 g/h·L. Third, STMβ is able to reach upto OD 4.01 in 32 hours. Last, the final ethanol yield from glucose of STMβ ($Y_{Ethanol/Glucose}$) is 0.39**.**

4.4 Single Copy Number of CDT and β-GL

Fig 4. Single copy number of CDT and single copy number of β-GL Fermentation Profiles

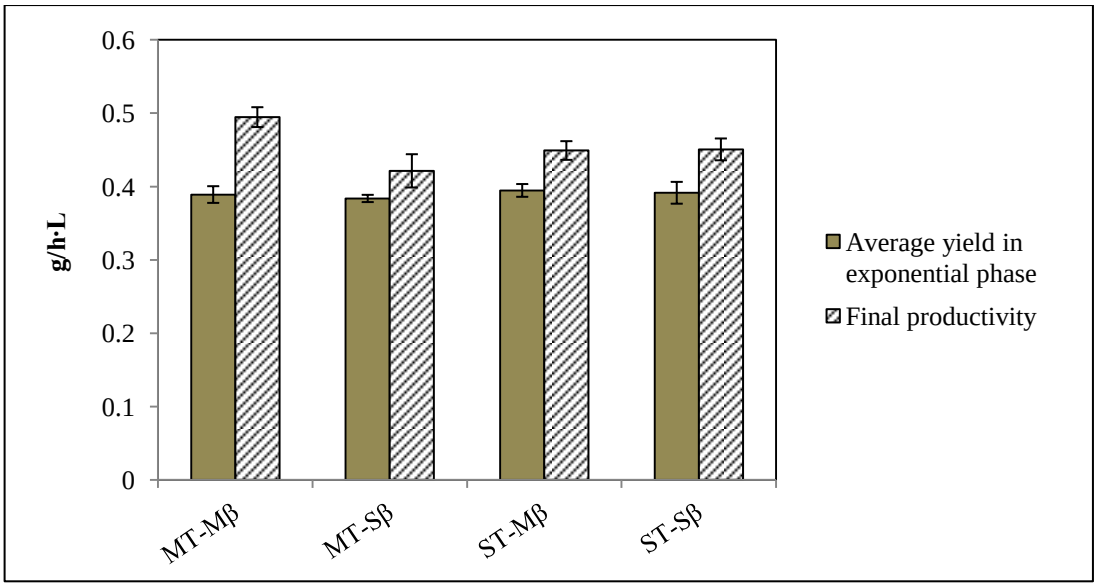


First, STSβ exhibited a capacity to consume 41.77 g/L of glucose within 35 hours, producing 15.77 g/L of ethanol. Second, the maximum volumetric productivity of glucose fermentation of STSβ is 0.50 g/h·L. Third, STSβ is able to reach upto OD 4.18 in 32 hours. Last, the final ethanol yield from glucose of STSβ ($Y_{Ethanol/Glucose}$) is 0.39.

4.5 Ethanol Yield and Fermentation Productivity

As the engineered strain, MTSβ showed better cell growth and ethanol fermentation, it seem that multi copy number of plasmids has a beneficial contribution to cell growth and ethanol fermentation. However, it seems that this improvement from both of multi copy number of plasmids is not very significant. Firgure 5 Showed average yield and final productivity of those four subjects. MTMB has 0.39 of the average yield in exponential phase and 0.49 g/h·L of final productivity. MTSβ has 0.38 and 0.42 g/h·L. STMβ has 0.39 and 0.45 g/h·L. STSβ has 0.39 and 0.45 g/h·L, respectively.

Fig 5. Ethanol Final productivity and average yield in exponential phase of 4 subjects



4.6 Summary of SCD Fermentation

Considering the potential benefits of more abundant amount of essential amino acids or nucleotides could help improve cell growth and ethanol fermentation on a genetic marker knock-out strain, it is not strange that MTMβ showed a slight faster rate

on cell growth and productivity. However, the improved ratio of the strains over the other three strains is 9 percentage in the average yield in exponential phase and almost same in final ethanol productivity. Thus, this improvement due to muli copy number of plasmids is not significant. Additionally, it is reasonably considered as a narrow and negligible gap because it is expected not to able to have significant effects under the very rich media such as YP media.

Chapter 5

Fermentation of Cellobiose with Four different Combinations of Cellodextrin CDT and β-GL in Copy Numbers Showed Different Yield and Productivity

As I observed previous preliminary experiments, the selection marker such as Urasil and Leucine did not have significant influence on cell growth and ethanol fermentation from a carbon source. Therefore, I assumed that these four engineered strains have no significant difference except only the copy number of the target genes. The aim of this section is to evaluate which gene has more contribution to production of ethanol from carbon source.

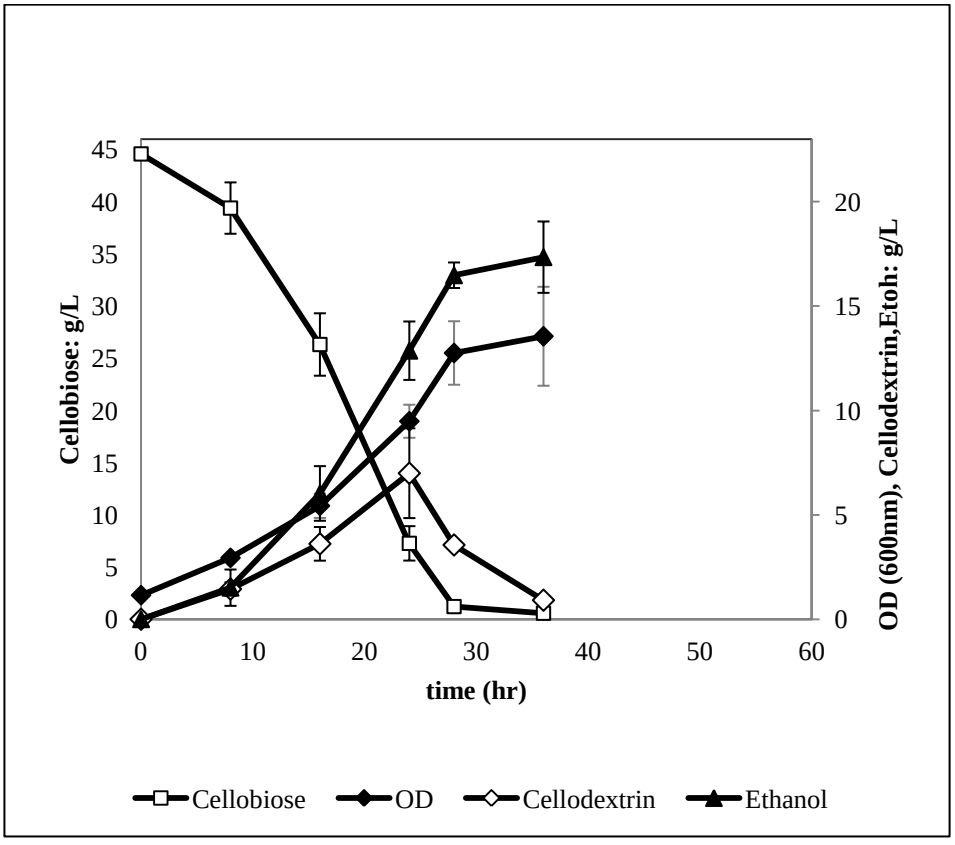
In order to measure which of CDT or β-GL is more critical on the cell growth and ethanol production, I focused on four features; the capacity to consume cellobiose within a given time; the volumetric productivity at the maximum state to produce ethanol; a final ethanol yield of the given strain; the highest amount of cellodextrin accumulation. Because I have hypotheses that the consumption of cellobiose can represent the general capabilitiy of the engineered strain, and the ethanol productivity and the ethanol yield can indicate the capacity of the strain to produce of ethanol. In addition, I presumed that cellodextrin can be an indicator of the efficiency to convert cellobiose to fementable carbon source. High amount of cellodextrin accumulation means β-GL converts cellobiose to non-fermentable carbon source, because β-GL can not only cut off cellobiose but also connect glucose to make cellodextrin such as dimer form of cellobiose or trimer for of cellotriose.

For the main culture, I put those strains into the yeast extract media which contains 2% of cellobios. Yeast extract and Pentptone media (YP media) has a general

purpose to grow a cell or strain, providing almost complete nutrientsor the cell confirm a complete transformation of plasmids by lacking selection markers such as essential amino acids or nucleotides. In addition, unlike confirmation of plasmids transformation, Yeast Peptone (YP) median is used as a fermentation medium for the main culture of cellobiose fermentation.

5.1 Multi Copy Number of CDT and β-GL

Fig 6. Multi copy number of CDT and multi copy number of β-GL Fermentation Profiles

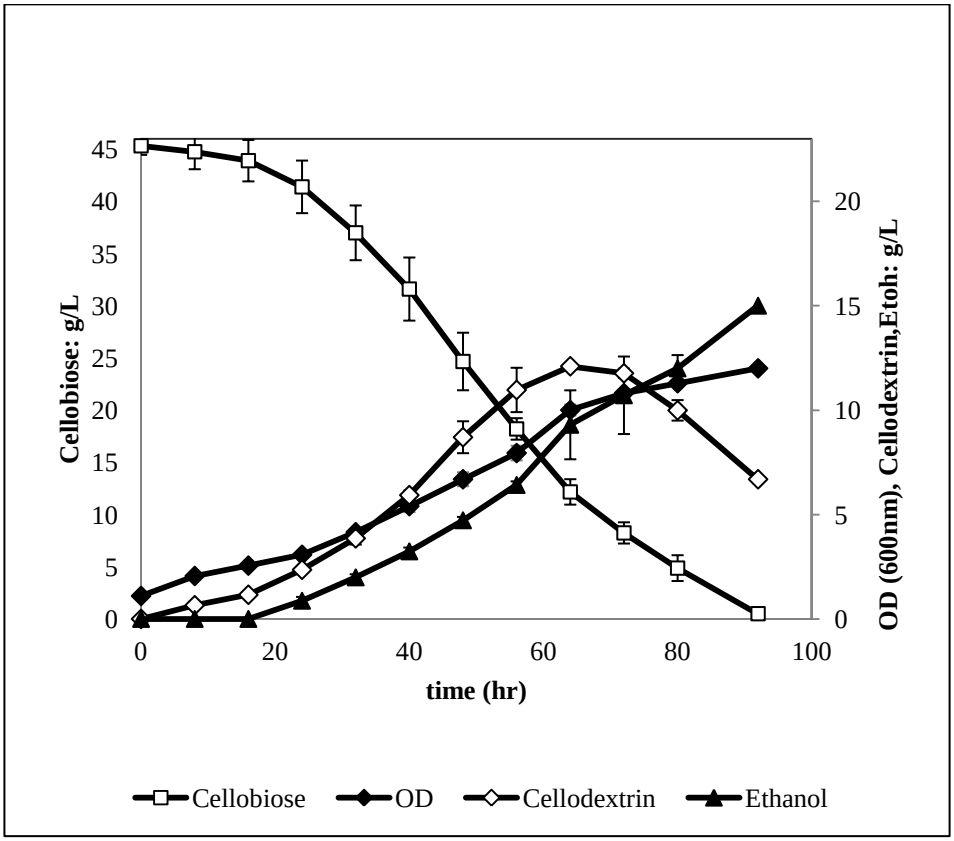


MTMβ exhibited the fastest fermentation rates. The transformant consumed 44.56 g/L of cellobiose within 26 hours, producing 17.34 g/L of ethanol. The volumetric productivity of cellobiose fermentation ($P_{Ethanol/Cellobiose}$= 0.67 g/h·L) was the most

efficient, and ethanol yield from cellobiose ($Y_{Ethanol/Cellobiose}$= 0.413).

5.2 Multi Copy Number of CDT and Single Copy Number of β-GL

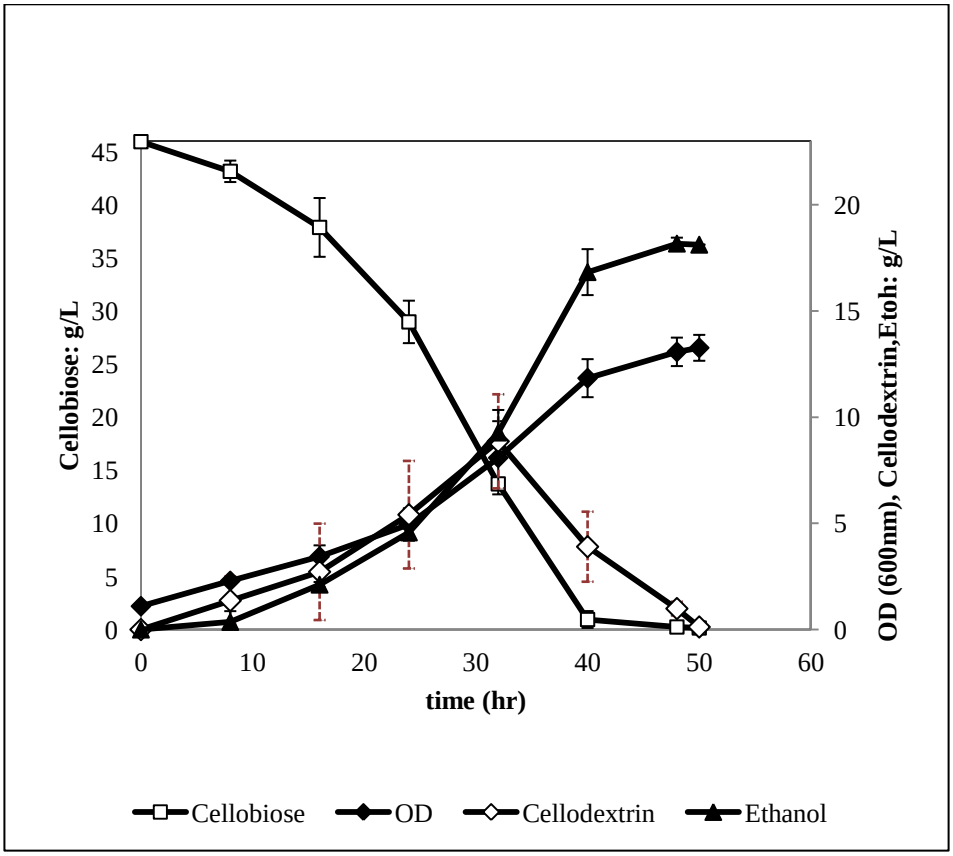
Fig 7. Multi copy number of CDT and single copy number of β-GL Fermentation Profiles



MTSβ exhibited the lowest cellobiose fermentation rates. The transformant consumed 45.31 g/L of cellobiose within 88 hours, producing 15.00 g/L of ethanol. The volumetric productivity of cellobiose fermentation ($P_{Ethanol/Cellobiose}$ = 0.393 g/h·L) was slower than those of STMβ, and ethanol yield from cellobiose ($Y_{Ethanol/Cellobiose}$= 0.40).

5.3 Single Copy Number of CDT and Multi Copy Number of β-GL

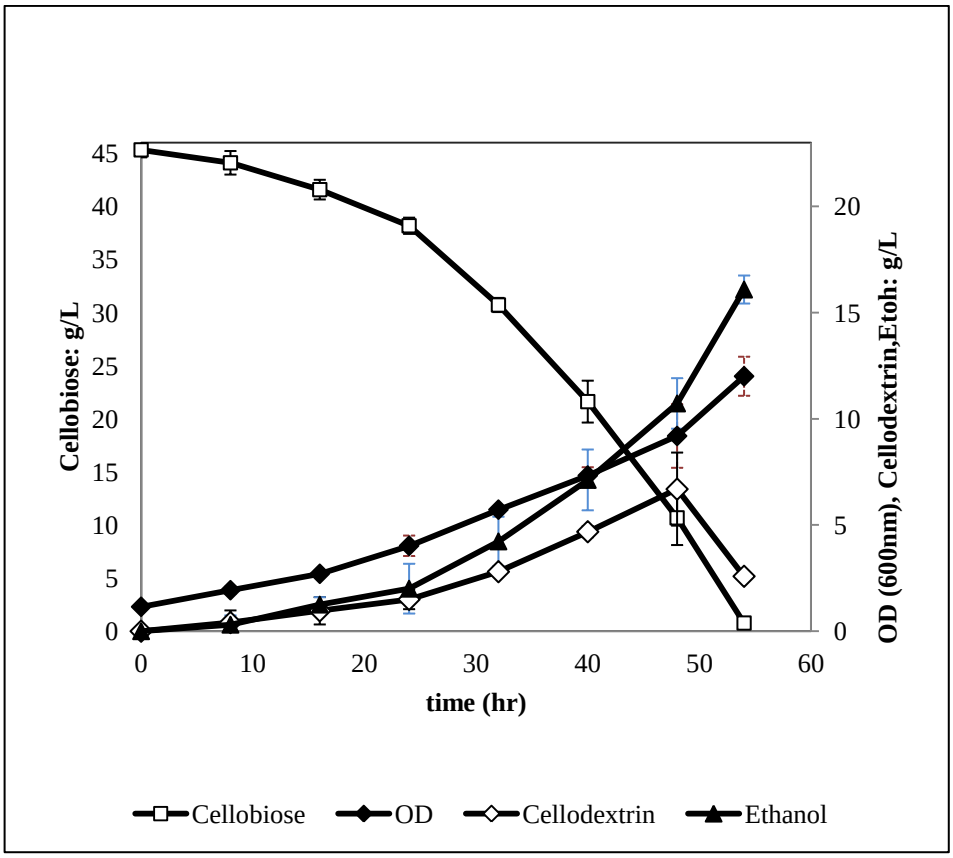
Fig 8. Single copy number of CDT and multi copy number of β-GL Fermentation Profiles



STMβ exhibited the second fastest cellobiose fermentation rates. The transformant consumed 45.93 g/L of cellobiose within 40 hours, producing 18.11 g/L of ethanol. The volumetric productivity of cellobiose fermentation ($P_{Ethanol/Cellobiose}$ = 0.397 g/h·L) was slower than those of MTMβ, and so was ethanol yield from cellobiose ($Y_{Ethanol/Cellobiose}$= 0.362).

5.4 Single Copy Number of CDT and β-GL

Fig 9. Single copy number of CDT and single copy number of β-GL Fermentation Profiles



STSβ exhibited the third fastest cellobiose fermentation rates. The transformant consumed 45.30 g/L of cellobiose within 54 hours, producing 16.08 g/L of ethanol. The volumetric productivity of cellobiose fermentation ($P_{Ethanol/Cellobiose}$ = 0.297 g/h·L) was the lowest, and ethanol yield from cellobiose ($Y_{Ethanol/Cellobiose}$= 0.3).

5.5 Both of Abundant Expression Levels in CDT and β-GL Lead to Increased Ethanol Productivity

Comparison of the fermentation patterns among those 4 different engineered *S. serevisiae* which contain multi copy number of CDT and β-GL plasmids reveals which one is the best performing strain for cellobiose fermentation. According to the experiment results, abundant expression levels of both cellodextrin CDT and β-GL guarantee significantly faster productivity ($P_{ethanol.Cellobiose}$= 0.588 g/h·L) and slightly betteryield ($Y_{Ethanol/Cellobiose}$ = 0.413) than other 3 strains. MTSβ showed $P_{ethanol.Cellobiose}$= 0.163 and $Y_{Ethanol/Cellobiose}$ = 0.407.STMβ showed $P_{ethanol.Cellobiose}$= 0.362 and $Y_{Ethanol/Cellobiose}$ = 0.400. STSβ showed $P_{ethanol.Cellobiose}$= 0.383 and $Y_{Ethanol/Cellobiose}$ = 0.298, respectively. Therefore, expression level of CDT and β-GL are important factors on cellobiose fermentation, and MTMβ is the strain to showed the best performance.

Fig 10.Ethanol Production

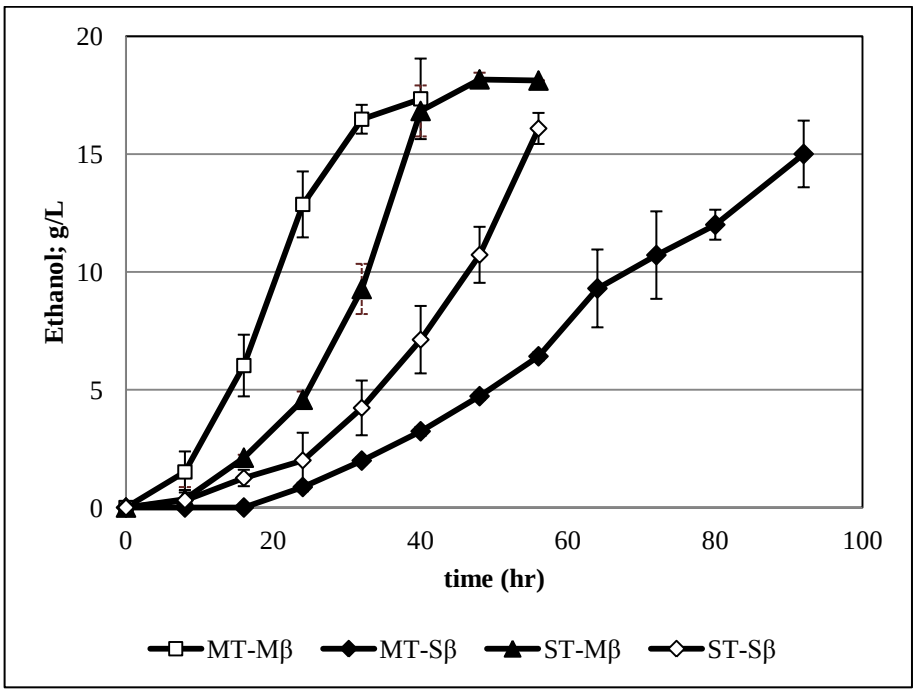
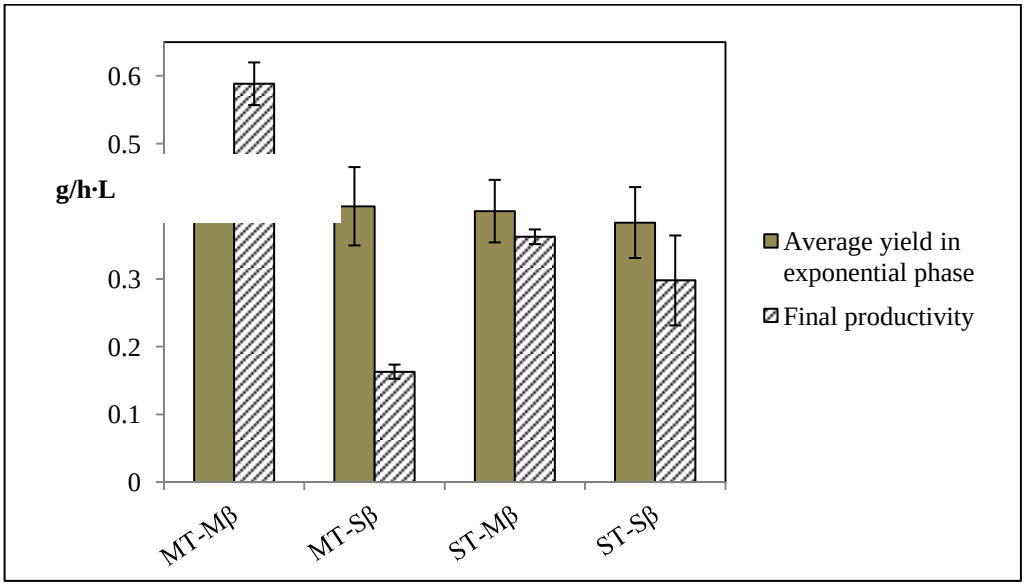


Fig 11.Total Ethanol Yield and Productivity



5.6 Limited Expression Levels in β-GL Lead to Detrimental Contribution on Ethanol Productivity and Efficiency

In order to discern which factors can have more significant influence on cellobiose fermentation, it is necessary to compare cellodextrin accumulation pattern. Because Cellobiose utilizing reactions are composed of two sequential reactions which are catalyzed by CDT and β-glucosidase, the accumulation of intermediate represents which expression level of factors is a critical point during the fermentation. Thus, a cellodextrin accumulation can be a potent indicator of capacity of cellobiose fermentation.

The maximum amount of cellodextrin accumulation in MTMβ is 6.99 g/L and it starts to be decreased after 24 hours. The maximum amount of cellodextrin accumulation in MTSβ is 12.10 g/L and it starts to be decreased after 64 hours. The maximum amount of cellodextrin accumulation in STMβ is 8.86 g/L and it starts to be

decreased after 32 hours. The maximum amount of cellodextrin accumulation in MTSβ is 6.69 g/L and it starts to be decreased after 48 hours.

According to these results, MTSβ showed extremely high amount (12.10 g/L) of cellodextrin accumulation and it is almost two fold of MTMβ cellodextrin accumulation. At the same time, the pattern of STMβ cellodextrin accumulation does not showed big difference from STSβ cellodectrs in accumulation. Thus, taken these two observations together, it suggest that if the strain did not secure enough amount of β-GL expression level, excessive expression level of CDT would have detrimental contribution on cellobiose fermentation.

Fig 12.CellodextrinAccumulation

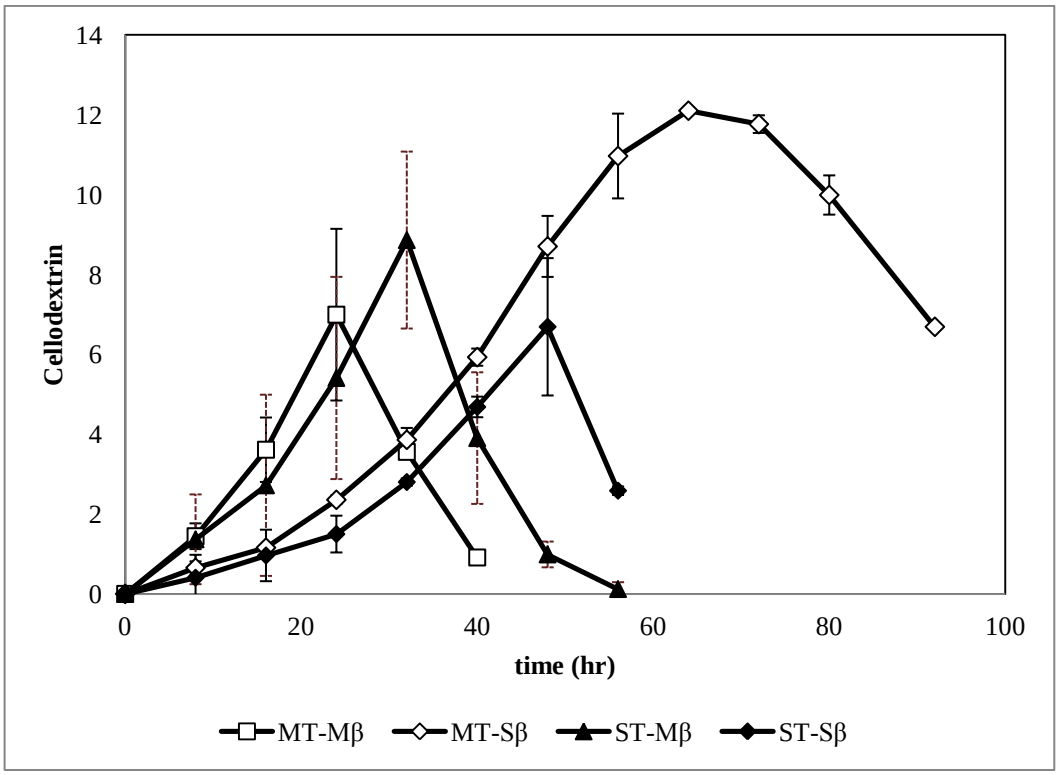
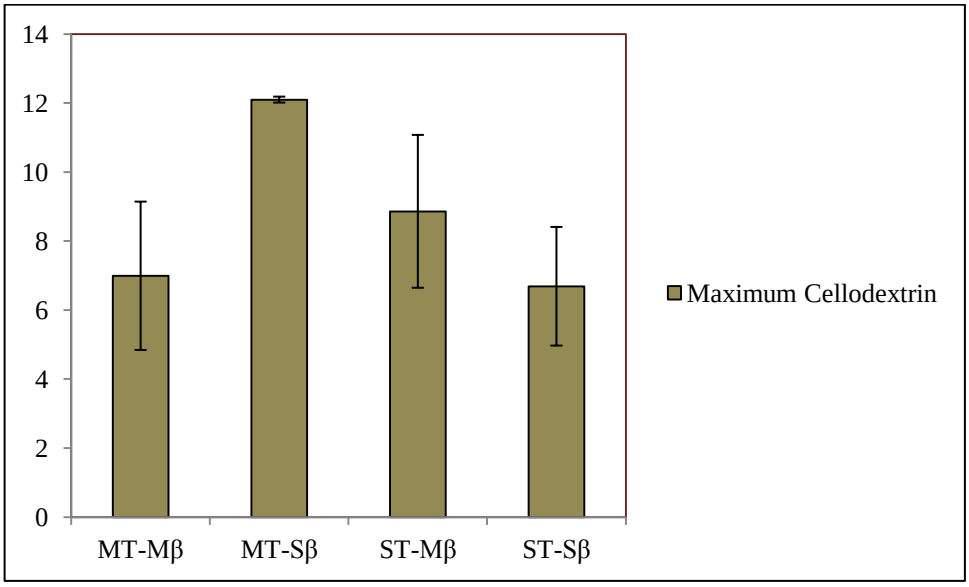


Fig 13. Maximum Accumulation of Cellodextrin



Chapter 6

Discussion

6.1 Changing Different Copy Number of Plasmids is an Effective Metabolic Engineering Method to Modulate the Ratio of given Enzymes

A metabolic engineering to change the copy number of plasmids, having certain genes on the sequence of reactions leading to one given process, is an effective method. Because expression level of plasmids replies on the copy number of plasmids, it can be an effective approach in metabolic engineering in a way to change quantities of enzymes and modulate the ratio of given enzymes. The whole processes of cellobiose fermentation in yeast are comprised of two major procedures which are cellobiose transportation and cellobiose hydrolyzation. In this study, modulation of expression levels which are involved with CDT and β-GL genes is used as a method to adjust the flux of those two steps. Coherent with original assumptions, the experimental results showed 4 different variations among those engineered strain having four different combinations of copy numbers in cellobiose transportation and hydrolyzation. Therefore, it is evident that metabolic engineering method to alter copy number of plasmids in the sequence of reactions leading to one given process is an operative and substantial strategy.

6.2 Both CDT and β-GL are considered as Important Factors in Cellobiose Fermentation

Some of enzymes are connected each other toward one sequence of reactions. For the effective metabolic engineering, it is essential to confirm the limiting enzyme

involved with the given reactions. In order to confirm how much impact each of those enzymes has, it is a good plan to assess the influence of the enzymes on final products or results in a way to modulate the flux of intermediate products. In this study, it is adopted as a measurement to alter a ratio of participation of given enzymes resulting in variations in final products by introduction of different combinations of copy number of plasmids. For example, different combinations of copy number of plasmids in CDT and β-GL engaged in cellobiose fermentation are introduced into *S. serevisiae* for altering flux catalyzed by these enzymes. As a result, all the transformants which introduced into 4 kinds of plasmids such as MTMβ, MTSβ, STMβ, and STSβ showed all different each other in fermentation profiles. It suggests that both of cellobiose CDT and β-GL are critical factors in cellobiose fermentation.

6.3 The Results suggest that β-GL could be a More Influencing Factor on Cellobiose Fermentation

To understand which enzyme has greater influence on cellobiose fermentation, it is necessary to compare each of those engineered strains. In this study, the comparison of experimental results leads two contradictory conclusions. For elucidating CDT effects, when comparing between STSβ and MTSβ, CDT seem like to have detrimental effects on fermentation. However, when comparing between STMβ and MTMβ, CDT imply to have beneficial effects on fermentation. But these two contradictory interpretations can reconcile when assuming sufficient participation of β-GL on cellobiose fermentation. In other words, with lack of β-GL, CDT make the series of reactions burden. But, with sufficient level of β-GL, CDT rather make the whole process accelerated. Thus, CDT can provide positive advantages into cellobiose fermentation as long as sufficient

quantity of β-GL is secured.

Chapter 7

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